Application of PCR, Real Time PCR and Loop mediated Isothermal amplification to detect *Orientia tsutsugamushi* DNA in Scrub Typhus antibody Positive and Negative patients and identification of genotypes circulating in and around Puducherry

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1. INTRODUCTION

Scrub Typhus (ST) is an emerging/re-emerging infectious zoonotic disease which occurs throughout the world and it is caused by *Orientia tsutsugamushi*, an obligate intracellular gram negative bacterium belonging to the member of family Rickettsiaceae. It is transmitted by the bite of infected larval stage (chiggers) of Trombiculid mites, *Leptotrombidium deliense* which are found in the areas of heavy scrub vegetation. ST is known for its endemicity in the so called "Tsutsugamushi Triangle" (Chattopadhyay et al., 2007) comprising many areas of North India, China, Japan, Indonesia, Malaysia, Thailand, Pakistan, Korea and northern Australia.

ST cases have been recently reported outside the triangle like Dubai, South America, Peru and Chile (Izzard et al., 2010; Weitzel et al., 2016; Balcell et al., 2011; Kocher et al., 2017). The illness starts from 6-21 days after mite bite (Rapsang et al., 2013). The most important clinical feature helpful in ST diagnosis is the appearance of a vesicular lesion called a black eschar. Other clinical symptoms like fever, headache, rash, lymphadenopathy etc. is inappropriate for ST findings, because these symptoms are also observed in other illness such as Dengue, Malaria, Leptospirosis, and other Haemorrhagic fevers. ST can also cause serious complications such as Gastrointestinal infections, Pneumonia, Myocarditis, Acute renal failure and Acute respiratory distress syndrome (Rapsang et al., 2013).

For isolation, it requires a expensive Bio-Safety Level III Containment facilities, and therefore it is not used as a routine method. In resource poor laboratories, Weil-Felix (WF) which is still performed with non-motile Proteus OXK antigens while it has low sensitivity. However, due to poor sensitivity and specificity, the use of WF is discouraged (Mahajan et al., 2006). Recently in India, ST diagnosis is being made by different serological tests like Enzyme Linked Immunosorbent Assay (ELISA) (Blacksell et al., 2016; Kocher et al., 2017; Stephen et al., 2016) Immunofluroescence assay (IFA) (Blacksell et al., 2016;

Rodkvamtook et al., 2013; Lim et al., 2015; Stephen et al., 2016) and Lateral flow assay based Rapid Immunochromatographic tests (ICT) (Lee et al., 2014; Stephen et al., 2015, Stephen et al., 2016; Anitharaj et al., 2016). The 'gold standard' IFA is expensive, needs high technical expertise and kits are to be imported. Therefore ELISA is recommended as an alternative to IFA (Blacksell et al., 2016; Koraluru et al., 2015; Pote et al., 2018).

More number of ST cases are missed due to the late appearance of antibodies detectable in the serological tests. This is overcome by new diagnostic assays viz., PCR, Nested PCR (N-PCR), Real Time PCR (qPCR) and Loop Mediated Isothermal Amplification (LAMP) which helps the early detection of *O. tsutsugamushi* DNA in blood samples. Efforts are made to increase the Diagnostic accuracy of ST by developing an algorithm 'Scrub Typhus Inclusion criteria (STIC) by Paris et al., 2011 and this was later modified by Lim et al., 2015 who lowered the IgM IFA significant titres from 1:12,800 to 1:3,200.

There are numerous outer membrane bacterial proteins (110, 80, 70, 60, 56, 47, 42, 35, 28, and 22kDa) present in *O. tsutsugamushi*. Among them, 56kDa occupied the 20% of protein (Ohashi et al., 1989). Usually 56kDa, 47kDa and groEL are the most commonly used target regions for PCR analysis (Lim et al, 2015).

LAMP is a new molecular method and easy to perform, with four to six primers in isothermal temperature and it is does not require costly instruments. LAMP is highly sensitive and specific, with both outer and inner primers designed for the detection of specific DNA in human clinical samples. To our knowledge, only few reports of LAMP for ST are available in world database (Paris et al., 2008, Huber et al, 2012; Thitimonrat Duangchanda 2012). In this study we have performed LAMP to identify *O. tsutsugamushi* targeting all three genes namely 56kDa, 47kDa and groEL.

Since more strain variations are commonly observed in *Orientia* 56kDa genome, there is a need to identify the prevalent genotype, which could also be useful later for vaccine

development. Among the various serotypes available for *O. tsutsugamushi*, Karp is the predominant genotype in worldwide (Kelly et al., 2009).

For treatment of scrub typhus doxycycline is considered to be the best effective drug, while azithromycin is the preferred choice for pregnant women and children below eight years (Chakraborty et al., 2017). In Paediatric ST, Clarithromycin would be recommended as a most effective drug when compared to chloramphenicol and azithromycin (Lee et al, 2017).

2. AIMS AND OBJECTIVES

Primary Objectives:

1. Confirmation of anonymised serum samples, which were ST IgM ELISA positive/negative for *O. tsutsugamushi* antibodies by application of PCR, Real Time PCR and LAMP.

2. Performing the gold standard IFA (IgM + IgG) and comparison with ELISA and molecular methods.

3. To analyze prevalent genotypes of *O. tsutsugamushi* in and around Pondicherry by gene sequencing. The study might reveal newer genotypes of *O. tsutsugamushi.*

Secondary Objectives:

1. Validating a commercial ST conventional PCR for 56kDa (Geno - sen's).

2. Validation of commercial ST qPCR (Geno - sen's) for targeting 56kDa gene.

3. Construction of LAMP primers for 56kDa gene.

3. BRIEF REVIEW OF LITERATURE

3.1. Global Prevalence of Scrub typhus:

ST is a re-emerging infectious disease, posing major health problems across the South Asia, North Asia as well as South East Asia and over a millions of cases are reported globally in every year. From the period of 1999, global outbreaks of ST cases were documented in various countries. In north Queensland from Australia 28 cases were reported among soldiers (Mc Bride et al., 1999). Recently another study from the same country reported 45 cases among military people (Harris et al., 2016). Maldives has reported 14 cases among 28 tested in Armed Forces Research Institute of Medical Sciences (AFRIMS, Bangkok) (Lewis et al., 2003), Papua New Guinea recorded 39 cases (Spicer et al., 2007). Rodkvamtook et al., 2013 reported 23 cases of ST in Northern Thailand among children. Durand et al., 2004 recorded 15 cases in Republic of Palau. Nine cases of ST were observed in the Western Province, Solomon Island (Marks et al., 2016).

Seroprevalence of ST varies within the endemic part of 'Tsutsugamushi triangle' including South East Asia and North Asian countries. The highest ST seropositivity of 68.4% was observed in Japan (Ogawa et al., 2008) followed by Thailand (59.5%) (Chanyasanha et al., 1998), North Vietnam (40.9%) (Hamaguchi et al., 2015). Moderate positivity was reported from Laos and Thailand among febrile patients, 20.3% (IgG antibodies) and 28.3% cases fulfilled the STIC criteria by Vallee et al., 2010 and Blacksell et al., 2015 respectively. A low positivity was reported from Combodia (3.7%) (Mueller et al., 2014) and Australia (0.6%) (Susilawati et al., 2015). Faa et al., 2006 reported that none of the patients studied by them were seropositive among pregnant women of Papua New Guinea.

Apart from tsutsugamushi triangle, ST seropositivity has extended to two countries, one from Djibouti region of Africa with three cases (Horton et al., 2016) and another from Kenya (Thiga et al., 2015).

3.2. Scrub typhus in India

ST is an endemic disease in India and is almost reported throughout like South, North, East and West (Rahi et al., 2015). During 2003 – 2016, a number of ST outbreaks has been reported in many states across India viz., Tamil Nadu, Himachala Pradesh, Manipur, Pondicherry, Meghalaya, Arunachala Pradesh, Andhra Pradesh, Rajasthan, Sikkim varying from 28 – 258 cases (Mathai et al., 2003; Gurung et al., 2013; Sharma et al., 2005; Krishna et al., 2005; Vivekanandan et al., 2010; Stephen et al., 2013; Stephen et al., 2015; Dass et al., 2011; Subbalaxmi et al., 2014; Sinha et al., 2014; Sharma et al., 2016; Usha et al., 2016). The positivity percentage of ST co-infected with other diseases such as Leptospirosis and Dengue was observed by different authors viz., 25% (Borkakoty et al., 2016), 28.5% (Gupta et al., 2016), Six and fifteen cases were found in (Basheer et al., 2016, Saleem et al., 2015).

Seroprevalence of ST was recorded among various states in India from 2004 onwards. From Vellore, Issac et al (2004) found one percent positivity among the control group in rural southern India. Sharma et al (2005) and Vaz et al (2006) reported a positivity of 34.7% and 50% in Weil Felix test for OXK antigen. Narvencer et al (2012) from Goa detected 34% by IgM ELISA and most of them were infected between July – November. During 2015, Roopa et al., 2015 from Pondicherry found 51(12.56%) of positivity when tested by Weil Felix. Ramyasree et al., 2015 from Andhra Pradesh confirmed 39% cases. Sengupta et al from Vellore in Tamil Nadu reported 15% of seroprevalence among blood donors, Khan et al., 2015 from Uttarkhand detected 19.4% by ICT and 59.5% by ELISA. In Karnataka, Koraluru et al (2015) reported that seropositivity of M-IFA, M-IFA + ELISA, M-IFA + Weil Felix, M-IFA + ELISA + Weil Felix were 26%, 22%, 18% and 16% respectively. Oberoi et al., 2015 from Punjab noted the sensitivity of 12.69% although the SD Bioline has poor sensitivity. Stephen et al, 2016 from Pondicherry found a sensitivity of 87% in ImmuneMed rapid kit, while Anitharaj et al., 2016 reported a sensitivity of 99.25%, 94.87%, 50.38%, against IgM

ELISA in InBios rapid kit, ImmuneMed kit and Weil Felix tests. Kalal et al (2016) noted 51.46% of sensitivity in Karnataka followed by Vinodkumar et al (2017) who reported positivity of 25.54% and 16.57% by Weil Felix and ELISA respectively. Jakharia et al (2016) had reported 40% of seroprevalence among farmers in seven districts in Arunachala Pradesh. Nelapati et al (2018) from Andhra Pradesh found 28.26% of seropositivity among febrile patients in both ICT and Weil Felix and a modest seropositivity of 25.5% and 23% were reported from Uttar Pradesh and Tamil Nadu (Rizvi et al and Jacob et al in 2018). In Mizoram, Lalrinkima et al (2018) found a seropositivity of 6.9% among 4083 samples tested.

4. MATERIALS AND METHODS

Type of Study: Prospective laboratory based study with Archived and anonymised blood samples

Place of Study:

1. Department of Microbiology, Mahatma Gandhi Medical College & Research Institute (MGMCRI) and

2. Department of Proteomics and Genomics, Centre for Interdisciplinary Research Facility (CIDRF), MGMC & RI Campus, Sri Balaji Vidyapeeth (Deemed-to-be-University), Puducherry.

Study Period: November, 2015 to March, 2018

Ethics approval:

Approval was obtained from our Institutional Human Ethical committee (IHEC), MGMC & RI.

Approval Project No: PG DISSERTATION/2015/10/01. Dated: 4th November 2015

Specimen Collection:

Sample size in each group: Based on the 85% sensitivity of the antibody tests, sample size (n) was calculated using the formula:

$$\text{“}n = \frac{1.96^2 (PQ)}{d^2}$$

Where confidence level at 95% (Standard value of 1.96)

P = Sensitivity of the antibody tests

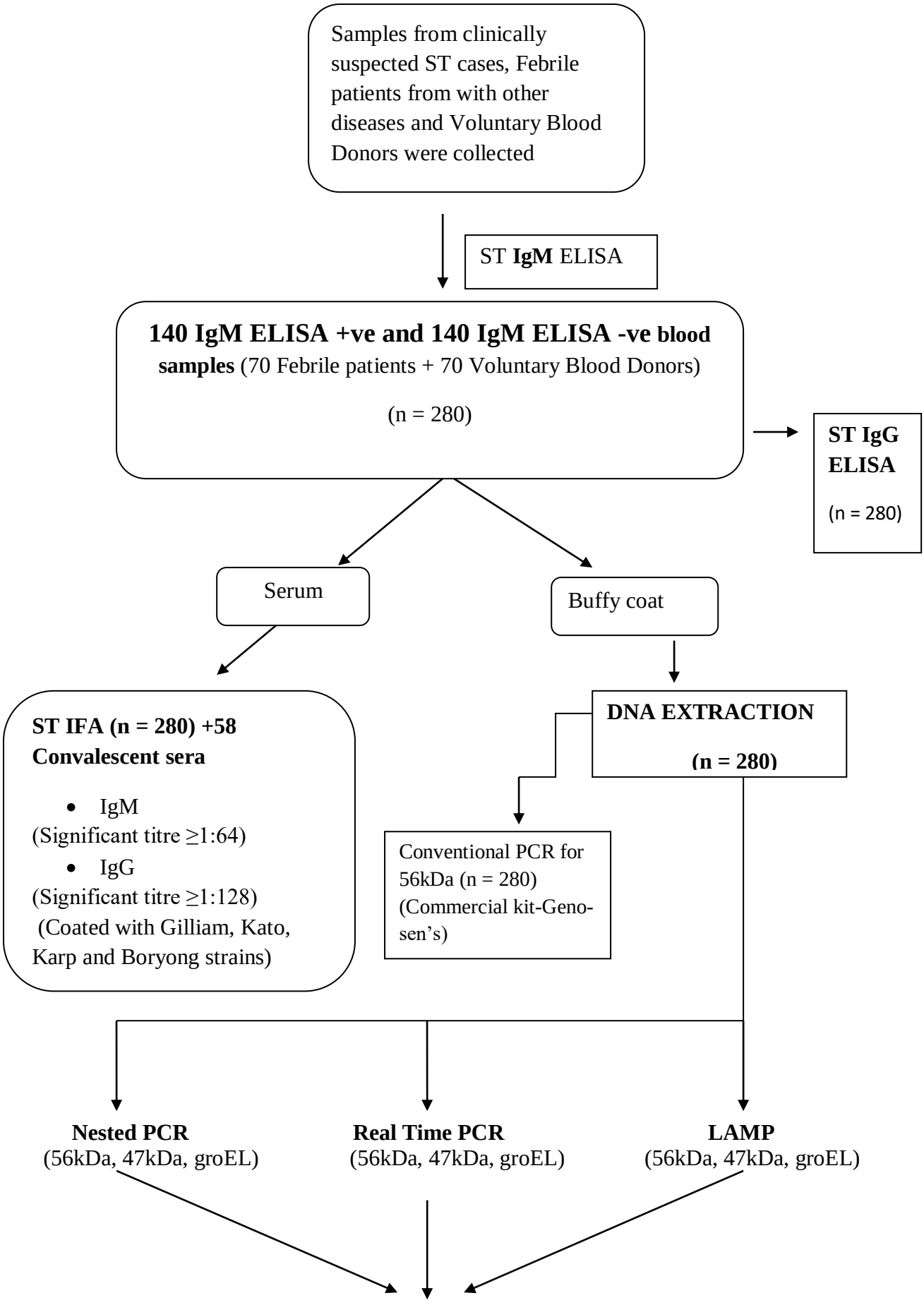
Q = 1-P

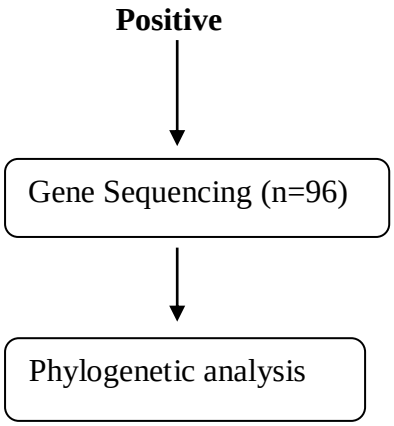
$\mathbf{d^2}$ = Margin of error at 6%

Antibody positive samples → 140* (Samples to be tested are 136, which is rounded upto 140)

Antibody negative samples →140 (70 Febrile patients with other diseases + 70 Voluntary Blood Donors)

After obtaining written informed consent from the patient, 7ml (2ml in plain tube+ 5ml in EDTA tube) of venous blood was collected. Buffy coat was prepared from EDTA treated samples and it was collected between the upper plasma layer and lower erythrocyte layer. Serum/ Buffy coat was separated and stored at -20ºc for further use. The flow chart of the procedure carried out for the molecular tests is as follows:


Samples from clinically suspected ST cases, Febrile patients from with other diseases and Voluntary Blood Donors were collected
ST IgM ELISA
140 IgM ELISA +ve and 140 IgM ELISA -ve blood samples (70 Febrile patients + 70 Voluntary Blood Donors)
(n = 280)
ST IgG ELISA
(n = 280)
Serum
Buffy coat
ST IFA (n = 280) +58 Convalescent sera
IgM
(Significant titre ≥1:64)
IgG
(Significant titre ≥1:128)
(Coated with Gilliam, Kato, Karp and Boryong strains)
DNA EXTRACTION
(n = 280)
Conventional PCR for 56kDa (n = 280) (Commercial kit-Geno-sen's)
Nested PCR
(56kDa, 47kDa, groEL)
Real Time PCR
(56kDa, 47kDa, groEL)
LAMP
(56kDa, 47kDa, groEL)



4.1. Serological Diagnosis of Scrub typhus:

Serological tests such as ELISA and IFA were performed for the detection of *Orientia tsutsugamushi* antibodies from serum samples.

IgM and IgG ELISA:

ST IgM ELISA was carried out for randomly selected scrub typhus cases and also for non scrub typhus cases (Febrile patients + Blood donors) using Scrub Detect TM IgM ELISA kit, InBios International, Seattle, USA. And for 140 IgM ELISA positive and 140 IgM ELISA negative samples ST IgG ELISA was also carried out using the same company kit. The procedure for both ELISA was in strict compliance with the manufacturer's instructions. An OD value of ≥ 0.5 for IgM ELISA (Stephen et al., 2013) and ≥ 1.8 (Varghese et al., 2018) for IgG ELISA were taken as positive.

Immunofluorescence assay:

ST IgM IFA and IgG IFA were performed for a total of 280 samples comprising of 140 antibody positive and 140 antibody negative samples. The kits were obtained from Fuller laboratories, California, USA and these slides were incorporated with four serotypes namely Kato, Karp, Boryong and Gilliam. The procedure was followed according to the technical brochure supplied in the kit. A small sharply defined fluorescent

rods within each antigen spot was taken as positive. To determine the highest end point dilution, positive samples were retested at varying dilutions. In negative reaction, only the counterstained cells were seen. As per the kit, a titre of 1:64 for IgM and 1:128 for IgG were considered as positive.

4.2. Molecular Diagnosis of ST:

DNA extraction:

DNA was extracted for a total of 280 samples as per the procedure of DNA Blood Mini Kit (Qiagen, Germany). The purity of the extracted DNA was checked by absorbance (A) using Eppendorf Spectrophotometer and the A260/A280 ratios observed in the range of 1.7-1.8 for all 280 samples. Finally the aliquoted DNA was stored at −80°C till the completion of the molecular tests.

Molecular tests such as Conventional PCR (56kDa) and Nested PCR, Real Time PCR, LAMP for the genes targeting 56kDa, 47kDa and groEL were performed for the detection of *Orientia tsutsugamushi.*

Commercial gel PCR for 56kDa:

Conventional PCR targeting 56kDa of *Orientia tsutsugamushi* was performed for a total of 280 samples using a commercial Geno – sen's gel PCR kit (Genome Diagnostics Pvt. Ltd, Solan, Himachal Pradesh, India). The test was carried as per the instructions of the kit's manufacturers.

Nested PCR:

ST Nested PCR was carried out for a total of 280 samples targeting three different genes namely 56kDa, 47kDa and groEL. The primers used for 56kDa, 47kDa and groEL were as described by Furuya et al., 1991; Kim et al., 2011 and Li et al., 2013

respectively. For all genes, first and second round of PCRs were performed with a total of 25µl reaction mixture but only the primers and template DNA were changed in the second round PCR. Apart from annealing temperature, the cycling conditions used were almost similar for the three genes. Finally the PCR products were electrophoresed in 1.5% agarose gel and the band according to the target were visualized by UV transilluminator (Gel Doc^{+}, Bio-Rad, USA).

Real Time PCR:

ST Real Time PCR was performed for a total of 280 samples targeting the same three genes in a Bio-Rad, CFX96 C1000 Touch machine. To amplify *Orientia tsutsugamushi* by the gene targeting 56kDa a commercial kit namely Geno – sen's Real Time PCR (Genome Diagnostics Pvt. Ltd, Solan, Himachal Pradesh, India) was used. For the detection for the other two genes namely 47kDa and groEL gene, an in house method was used. For 47kDa (TaqMan based) primers and probe were used from Kim et al, 2011 and for groEl the primers were selected as per the report of Paris et al., 2009. In each run a positive and negative controls were included. All samples were run in duplicates for confirmation of result. A cq value of ≤ 35 was considered as positive. To ensure the quality of DNA, an internal control namely Human β – actin (ACTB) was used for every DNA sample.

Loop Mediated Isothermal Amplification (LAMP):

For a total of 280 samples ST LAMP was performed targeting all three genes. We have designed primers for LAMP targeting 56kDa of *Orientia tsutsugamushi* using Hualien – 5 strain (Accession no. AY243357.1). For 47kDa and groEL gene the primers were used as suggested by Kim et al., 2011 and Paris et al., 2008 respectively. A total of 25µl of reaction volume contains 10 X Isothermal amplification buffer II, Primers, Betaine, $MgSO_4$, DNTPs and also Hydroxy Naphthol blue was added for colour formation. Within one hour the amplification was completed at a constant temperature with four to six primers, (Loop

primers-2). As a result the colour changes from violet to sky blue were taken as positive and no changes in colour were taken as negative.

Gene Sequencing:

In the present study, totally 96 (45 - groELgene,41 - 56kDa gene and 10 – 47kDa gene) nested PCR products were purified using QIA quick PCR purification kit (Qiagen, Germany) as per the procedure outlined by the manufacturers. The purified products were confirmed in a 2.0% agarose gel electrophoresis stained with Ethidium bromide. These amplicons were custom sequenced by means of bidirectional single pass sequence analysis by an Applied Biosystems, ABI3730XL.

5. STATISTICAL ANALYSIS

Percentages were calculated for categorical variables. Kappa agreement was calculated for different diagnostic tests and compared using Chi-square test or Fisher's exact test for small number of samples. P values < 0.05 were considered statistically significant.

6. RESULTS

Among 184 Serological/Molecular confirmed scrub typhus cases, 64 (34.78%) were children and 120 (65.22%) were adults. Out of 64 children 35 (19.02%) were male and 29 (15.76%) were female. Out of 120 adults, 78 (65%) were male and 42 (22.83%) were female.

6.1. ST ELISA:

6.1.1. IgM ELISA:

A total of 140 antibody positive samples and 140 antibody negative samples (70 Febrile patients + 70 Blood donors) were included in the study for further molecular confirmation.

6.1.2. IgG ELISA:

Out of 280 samples tested, 65 (23.21%) were positive and 215 (76.78%) were negative for IgG ELISA. Among 140 IgM ELISA positive samples, 52 were IgG ELISA positive and 88 were IgG ELISA negative. However in 140 IgM ELISA negative samples, 13 were IgG ELISA positive and 127 were IgG ELISA negative.

6.2. Immunofluorescence assay

6.2.1. IgM IFA:

Overall positivity observed in IgM IFA was 136 (48.57%) which comprises of 120/140 ELISA positive and 16/140 ELISA negative. Table – 1 shows the results of various combinations IgM IFA positivity of four serotypes. The total ST IgM IFA positivity of different serotypes were Kato – 89, Karp – 80, Boryong – 97, Gilliam – 130.

Table – 1: ST IgM IFA Positivity of four serotypes (Kato, Karp, Boryong and Gilliam) in various combinations.

SL.no	Combinations of ST IgM IFA	ST IgM ELISA Positive (n = 140)	ST IgM ELISA negative (n = 140)	Total (n = 280) (%)
1	Kato + Karp + Boryong + Gilliam	60	4	64 (22.85)
2	Karp + Kato + Boryong	2	1	3 (1.07%)
3	Karp + Boryong + Gilliam	4	-	4 (1.42%)
4	Boryong + Gilliam + Kato	7	1	8 (2.85%)
5	Gilliam + Kato + Karp	5	-	5 (1.78%)
6	Kato + Gilliam	7	1	8 (2.85%)
7	Karp + Gilliam	3	1	4 (1.42%)
8	Boryong + Gilliam	14	2	16 (5.71%)
9	Kato only	1	-	1 (0.5%)
10	Boryong only	2	-	2 (0.71%)
11	Gilliam only	15	6	21 (7.5%)
12	Total positive	120	16	136 (48.57%)
13	All Negative	20	124	144 (51.42%)
	Total	140	140	280

6.2.2. IgG IFA:

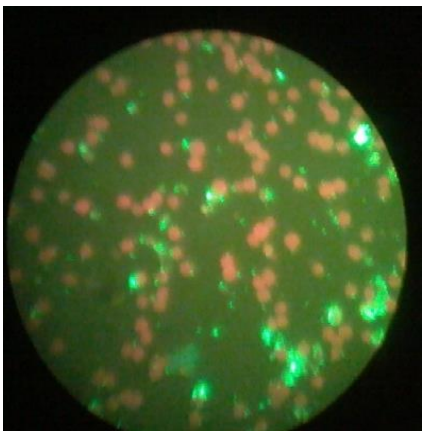
Among 65 IgG ELISA positive and 215 ELISA negative, 30 (46.15%) and 38 (17.67%) were positive for IgG IFA respectively. The total ST IgG IFA positivity of different serotypes were Kato – 45, Karp – 36, Boryong – 43, Gilliam – 65. Table – 2 shows the results of various combinations IgM IFA positivity of four serotypes. Images of positive and negative results of IgM and IgG IFA were shown in Figure – 1.

Table – 2: ST IgG IFA Positivity of four serotypes (Kato, Karp, Boryong and Gilliam) in various combinations.

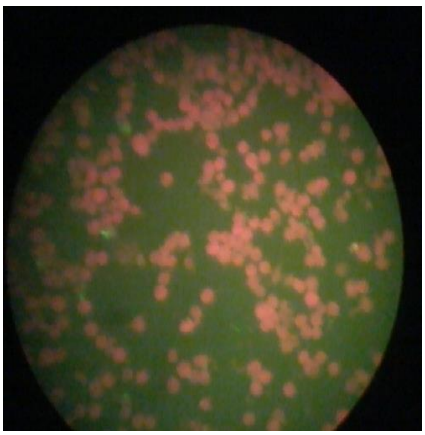
SL.no	Combinations of ST IgG IFA	ST IgG ELISA Positive (n = 65)	ST IgG ELISA negative (n = 215)	Total (n = 280) (%)
1	Kato + Karp + Boryong + Gilliam	6	11	17 (6.07%)
2	Karp + Kato + Boryong	1	-	1 (0.35%)
3	Karp + Boryong + Gilliam	-	2	2 (0.71%)
4	Boryong + Gilliam + Kato	4	2	6 (2.14%)
5	Gilliam + Kato + Karp	6	6	12 (4.28%)
6	Kato + Karp	1	1	2 (0.71%)
8	Kato + Gilliam	-	2	2 (0.71%)
10	Karp + Gilliam	-	2	2 (0.71%)
11	Boryong + Gilliam	6	11	17(6.07%)
15	Gilliam only	6	1	7 (2.5%)
16	Total positive	30	38	68 (24.29%)
17	All Negative	35	177	212 (75.71%)
	Total	65	215	280

Figure – 1: Results of ST IgM IFA

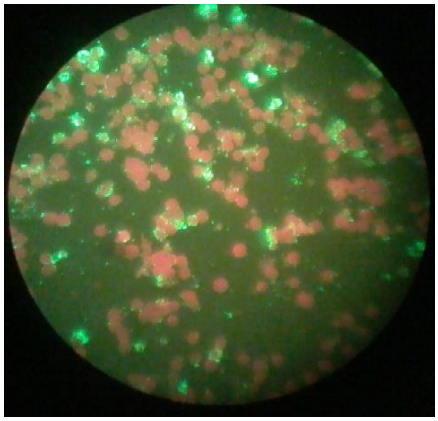
1.1. IgM IFA Positive control → Boryong



1.2. IgM IFANegative control →Boryong



1.3. Positive for Karp (IgM IFA→1:64)



1.4. Negative for Karp (IgM IFA →1:64)

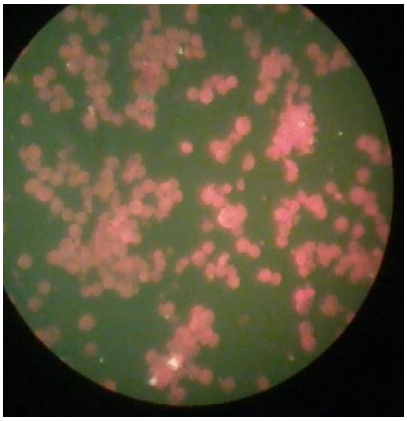
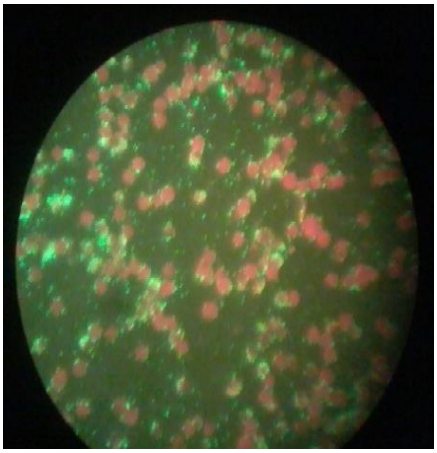


Figure – 1: 1.1– Positive control; 1.2 – Negative control; 1.3 – Sample positive for IgM IFA;

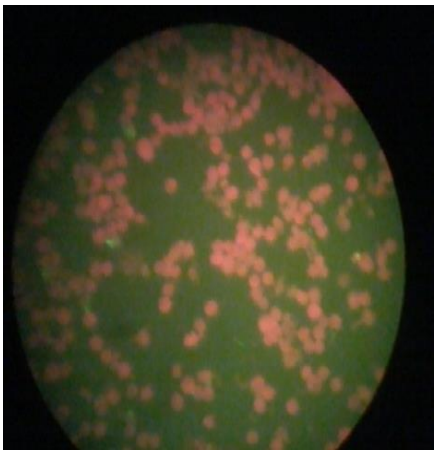
1.4 - Sample negative for IgM IFA;

Figure – 2: Results of ST IgG IFA

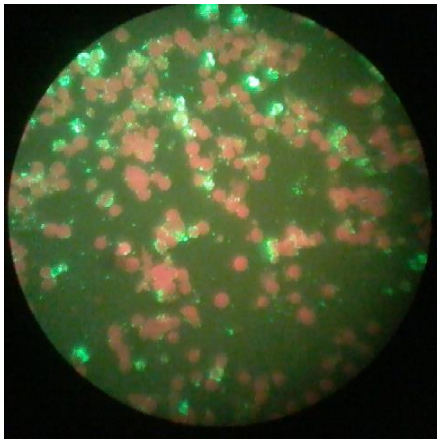
2.1. IgG IFA Positive control → Gilliam



2.2. IgG IFA Negative control → Gilliam



2.3. Positive for Kato (IgG IFA →1:128)



2.4. Negative for Kato (IgG IFA →1:128)

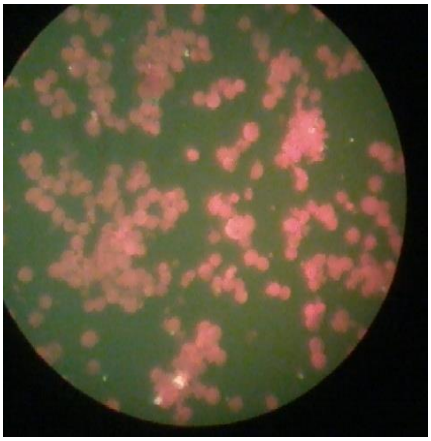


Figure – 2: 2.1- Positive control; 2.2- Negative control; 2.3 - Sample positive for IgG IFA; 2.4 – Sample negative for IgM IFA

6.3. Paired sera in ELISA (IgM and IgG) and IFA (IgM and IgG):

Among 58 paired sera, all 58 were IgM ELISA positive in acute stage of ST, but in convalescent stage only 50 were IgM ELISA positive and eight were IgM ELISA negative. Regarding IgG ELISA, 25 were positive and 33 were negative in acute conditions whereas 38 were positive and 20 negative among convalescent samples. IFA IgM was positive in 49 and negative in nine acute samples. Regarding convalescent samples, 39 out of 50 ELISA positive sera were IgM IFA positive and out of eight ELISA negative convalescent samples three were IgM IFA positive.

Among 58 acute samples in paired sera, IgM ELISA positivity was observed in all 58 cases but IgG ELISA positivity was observed in 35 only. Regarding IFA, 49 were positive in IgM IFA and 33 were positive in IgG IFA.

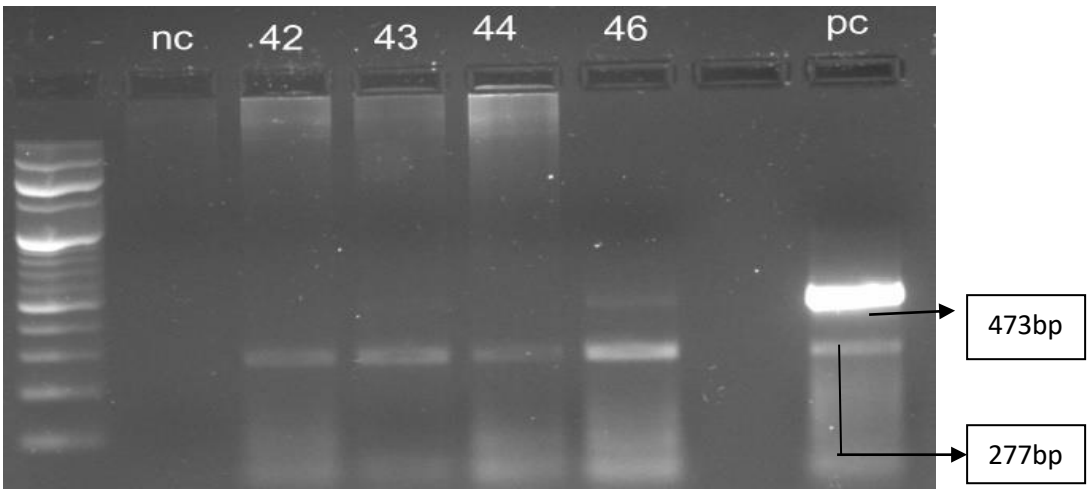
In a total of 58 convalescent samples in paired sera, 50 were positive in IgM ELISA but IgG ELISA positivity was observed in only 38. While in IFA, 42 were IgM IFA positive but only 38 were IgG IFA positive.

Note : All 70 blood donors were negative for *O.tsutsugamushi* antibodies in serological methods such as ELISA and IFA.

6.4.Commercial gel PCR for 56kDa:

Among 140 IgM ELISA positive samples, 25 were positive (17.86%) in this commercial PCR and none of them were positive in the ELISA negative group. Out of 25 positive in conventional PCR, 22 were IgM IFA positive and three were negative. The gel picture with internal control (to access the quality of DNA) is shown in figure – 3.

Figure –3: Conventional PCR for 56kDa



Lane 1 – 100bp DNA Ladder; nc - Negative control; 42 – 44 → Sample negative; 46- →Sample positive for *Orientia tsutsugamushi.*; pc – Positive control at 473bp;

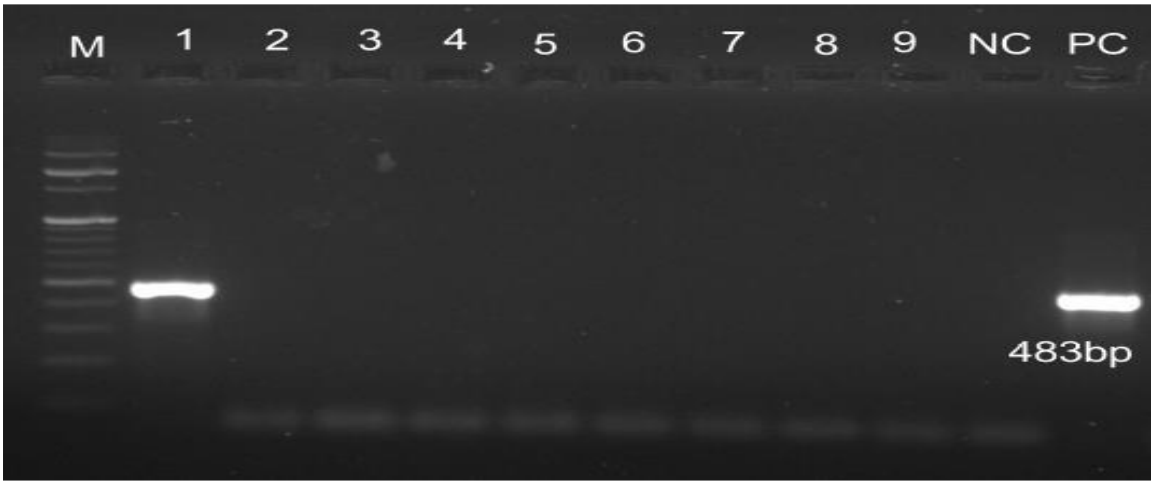
6.5. NESTED PCR:

In Nested PCR, a total of 45/280 (16.07%) were positive for the gene targeting 56kDa which shows the band at 483bp (Figure – 4) in gel. Followed by 76/280 (27.14%) in 47kDa and 89/280 (31.78%) in groEL were positive with bands at 118bp (Figure – 5) and 364bp (Figure – 6) respectively. The positivity observed in only one gene was 56kDa – 4; 47kDa – 11; groEL – 24. Twenty three of them were positive in all three genes. Out of 280, overall positivity observed in any one/two/three genes was 113/280 (40.36%). The Nested PCR positivity for three genes with reference to ELISA and IFA is shown in figure – 7.

Statistical analysis:

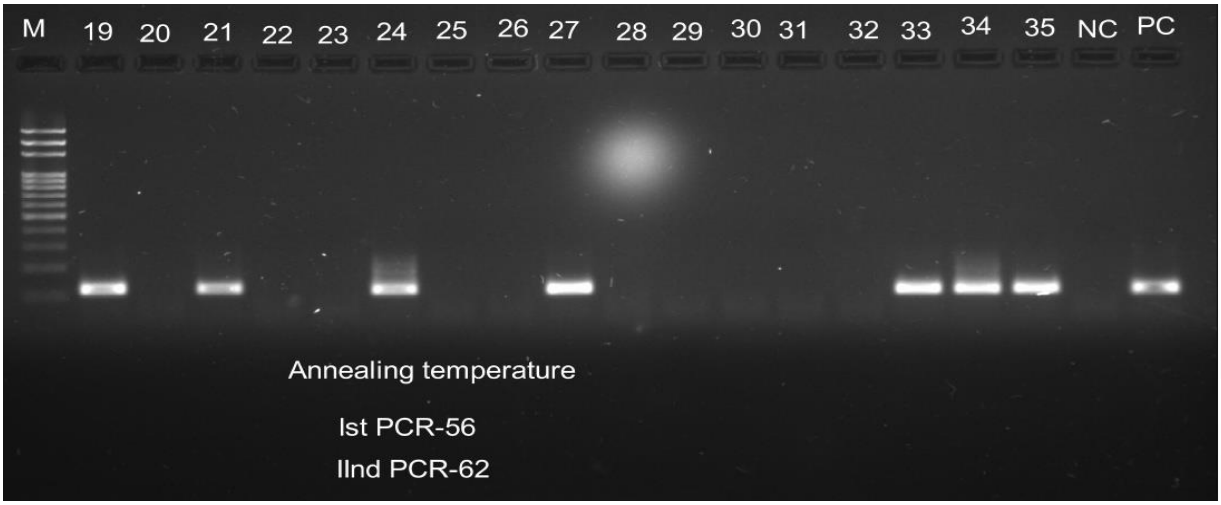
p value for Positivity between ELISA Positive and Negative samples as well as for IFA Positive and Negative samples in all three genes of Nested PCR are statistically significant as detailed in Table – 3.

Figure – 4: Nested PCR for 56kDa:



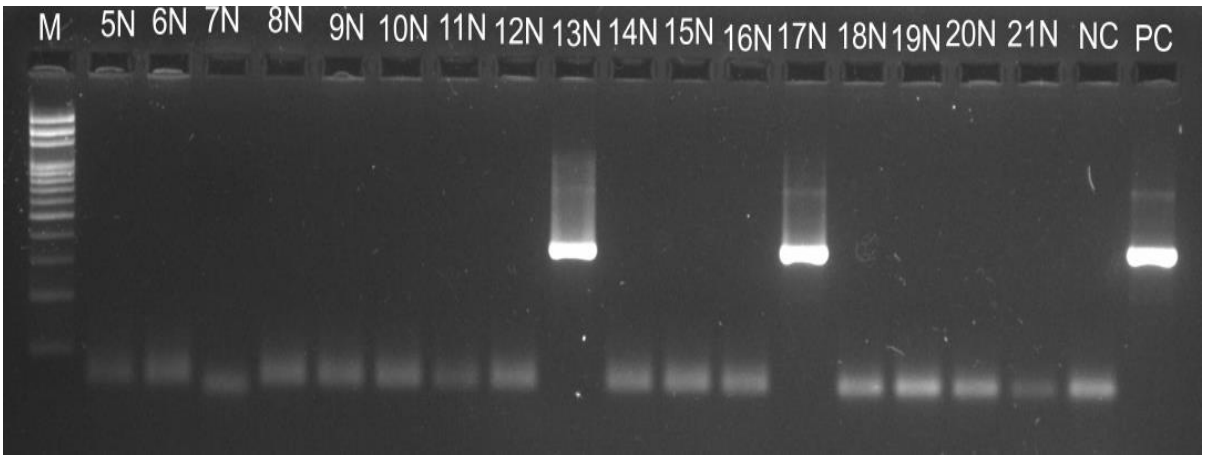
M – 100bp DNA Ladder, 1- Positive sample, 2→9 – Negative sample, NC- Negative control, PC – Positive control.

Figure – 5: Nested PCR for 47kDa:



M – 100bp DNA Ladder; Samples no 19,21,24,27,33,34,35 showing the bands at 118bp were positive; NC – Negative control and PC – Positive control

Figure – 6: Nested PCR for groEL gene:



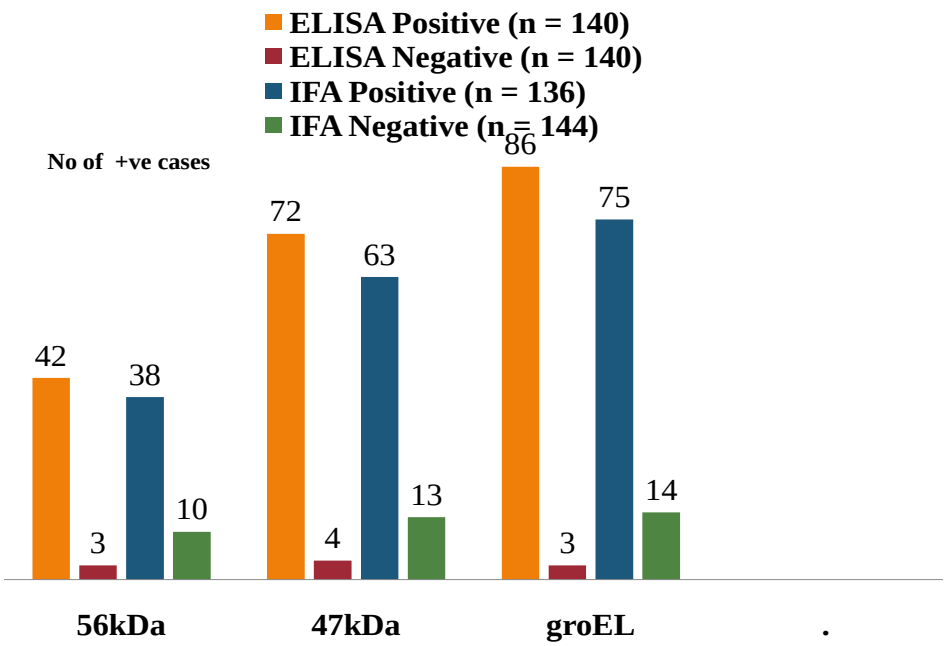
M – 100bp DNA Ladder; 13N and 17N → Test positive; 5N-12N, 14N-16N and18N-21N – Test Negative; NC – Negative control and PC – Positive control at 364bp

Table – 3: Positivity of 56kDa/47kDa/groEL in Nested PCR, ELISA and IFA (n =280)

Name of the gene targets	Nested PCR				Nested PCR			
	ELISA Positive (n = 140)	ELISA Negative (n = 140)	Total	p value*	IFA Positive (n = 136)	IFA Negative (n = 144)	Total	p value*
56kDa	42	3	45	**0.0001**	37	8	45	**0.0019**
47kDa	72	4	76	**0.0001**	64	12	76	**0.0006**
groEL	86	3	89	**0.0001**	75	14	89	**0.0025**

* p value <0.05 was considered as statistically significant

Figure – 7: Results of ELISA and IFA in Nested PCR for three genes (56kDa/47kDa / groEL) of *Orientia tsutsugamushi*.



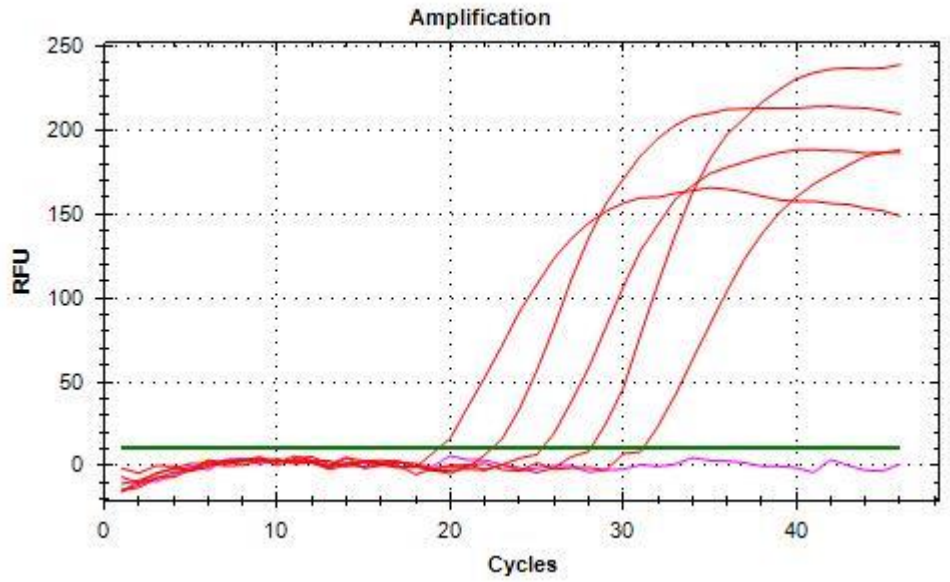
6.6. REAL TIME PCR:

A total positivity of real time PCR targeting 56kDa, 47kDa and groEL gene observed were 85/280 (30.35%), 75/280 (26.78%) and 102/ 280 (36.42%) respectively. Overall positivity of real time PCR targeting 56kDa/47kDa/groEL was 151/280 (53.93%). For quantification purpose, a standard curve was performed with varying concentration of known positive DNA for the genes (Figure – 8). DNA controls for the four genotypes were very kindly supplied by Dr. Sunghyuk choi, ImmuneMed, South Korea. Twenty six out of 280 samples were positive for all three genes of *O. tsutsugamushi.* Positivity observed in only one gene was 56kDa – 23, 47kDa – 14, groEL – 29. Positivity of Real Time PCR in ELISA Positive/Negative and IFA Positive/ Negative were shown in figure – 9.

Statistical analysis:

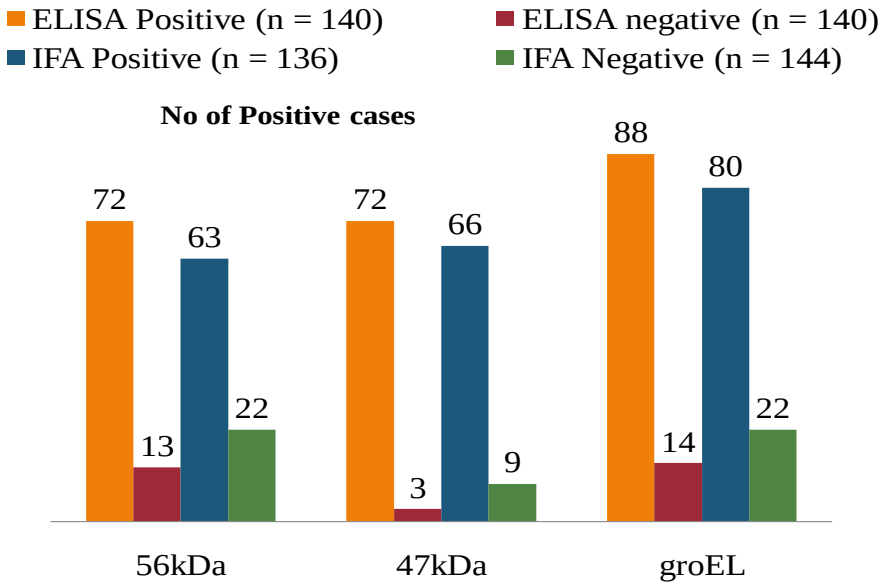
p value for Positivity between ELISA Positive and Negative samples as well as for IFA Positive and Negative samples in all three genes of Real Time PCR are statistically significant (Table – 4).

Fig – 8: Standard curve for Real Time PCR:



1. Magenta → 10^5 - 10^1 opies of DNA; 2. Pink – Negative control

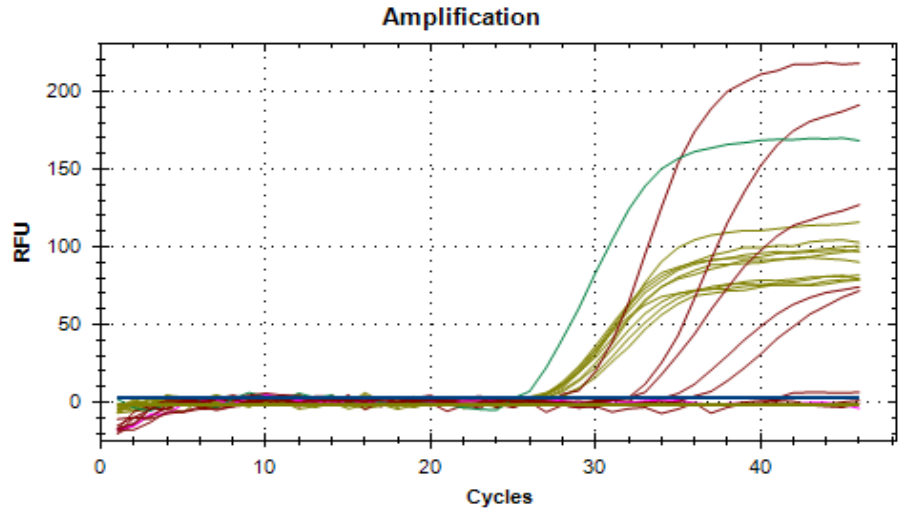
Figure – 9: Results of ELISA and IFA in Real Time PCR for three genes (56kDa/47kDa / groEL) of *Orientia tsutsugamushi*.



Amplification curves for 56kDa, 47kDa and groEL

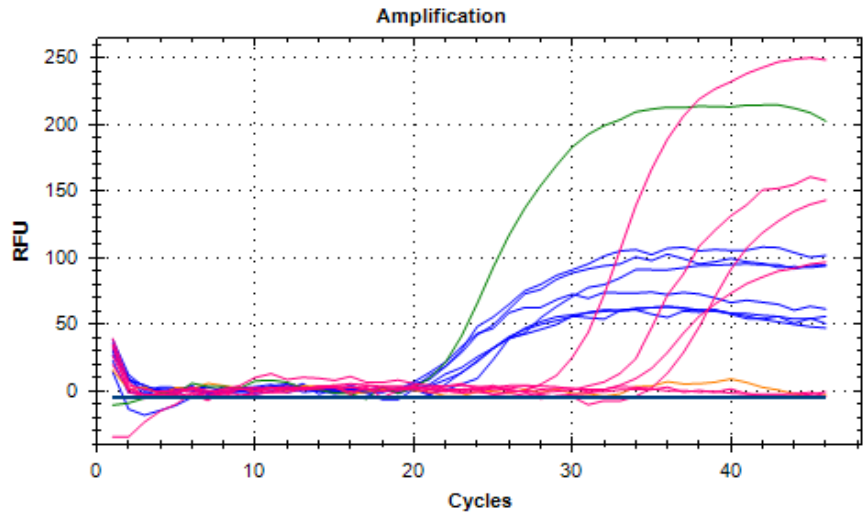
The amplification curve obtained for 56kDa (Commercial kit), 47kDa (In house PCR – TaqMan based), groEL (In house PCR – SYBR green based) is shown in figure – 10, 11 and 12.

Figure – 10: qPCR for 56kDa (Geno - sen's commercial kit)



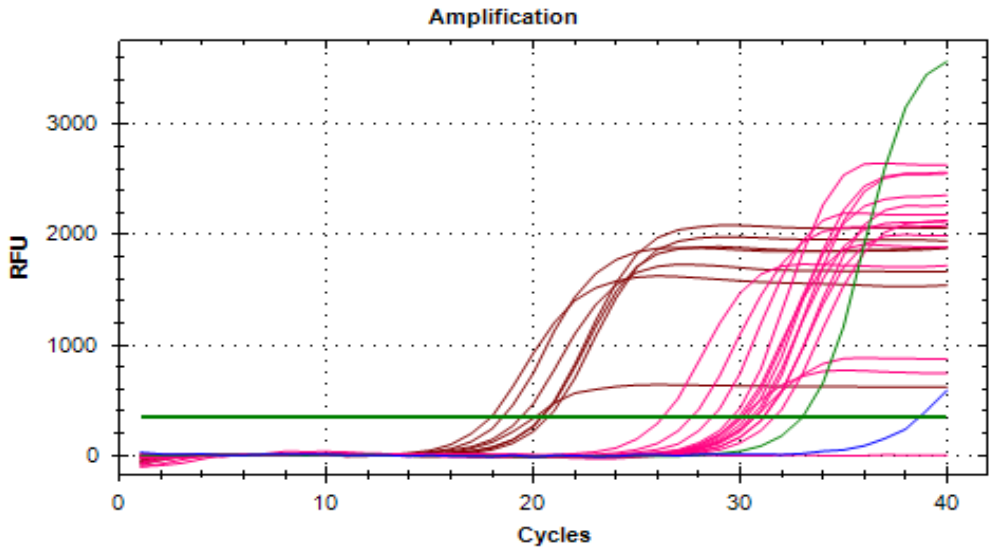
Green – positive control; Blue – Negative control; Light green – Internal control (β – actin); Magenta - samples

Figure – 11: qPCR for 47kDa (Taqman probe based assay)



1. Green - Positive control ; 2. Orange - Negative control ; 3. Pink – Positive Samples; 4. Blue - Internal control (β- actin).

Figure – 12: qPCR for groEL gene:



1. Green - Positive control 2. Blue - Negative control 3. Pink – samples 4. Brown - Internal control (β – actin)

Table – 4: Positivity of 56kDa/47kDa/groEL for Real Time PCR in ELISA and IFA (n =280)

Name of the gene targets	Real Time PCR				Real Time PCR			
	ELISA Positive (n = 140)	ELISA Negative (n = 140)	Total	p value*	IFA Positive (n = 136)	IFA Negative (n = 144)	Total	p value*
56kDa	72	13	85	**0.0001**	63	22	85	**0.0485**
47kDa	72	3	75	**0.0001**	66	9	75	**0.0002**
groEL	88	14	102	**0.0052**	80	22	102	**0.0305**

* p value <0.05 was considered as statistically significant

6.7. Loop Mediated Isothermal Amplification

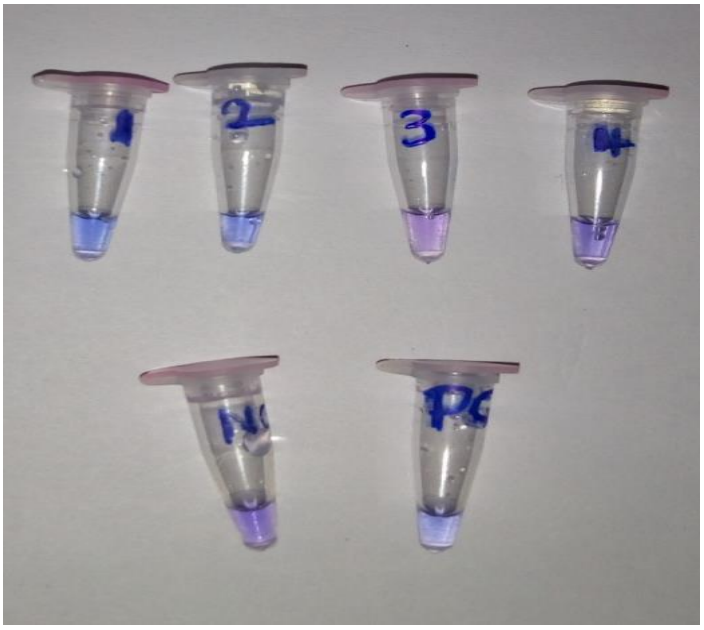
LAMP was performed for a total of 280 samples targeting the three genes such as 56kDa, 47kDa and groEL genes of *O. tsutsugamushi*. LAMP result can be visually monitored by colour change from violet to sky blue as shown in Figure – 13. Positivity of LAMP for the genes were - 102 for 56kDa (36.43%), 83 for 47kDa (29.64%) and 95 for groEL (33.93%). Overall positivity of LAMP targeting 56kDa/47kDa/groEL was 54.29% (152/280). Among 280, 34 were positive in all three genes. Only one gene positivity was observed in 56kDa, 47kDa and groEL were 24 (8.57%), 11(3.93%) and 23(8.21%) respectively. Positivity of LAMP in ELISA Positive/Negative and IFA Positive/ Negative were shown in histogram (Figure – 15). The results were also confirmed in gel by ladder like pattern appearance (Figure – 14).

Statistical analysis:

Regarding ELISA positive and negative samples against LAMP targeting 56kDa/47kDa/groEL genes was found to be statistically significant ($p<0.05$) (Table – 5). Similar comparison is made for IFA positive and negative samples vs LAMP. There was no statistical difference was made between IFA positive and negative samples for 56kDa and groEL gene but 47kDa was statistically significant ($p<0.05$) (Table – 5).

Note : All 70 blood donors were negative in all molecular methods -Nested PCR, Real Time PCR and LAMP- targeting all three genes of *O.tsutsugamushi*.

Fig – 13: Visual Detection of LAMP



Upper row: 1 & 2 – Samples positive for *O.tsutsugamushi* DNA, 3 and 4 –Sample negative for *O.tsutsugamushi* DNA; **Lower row:** NC – Negative control, PC – Positive control.

Figure – 14: Analysis of LAMP products in agarose gel electrophoresis

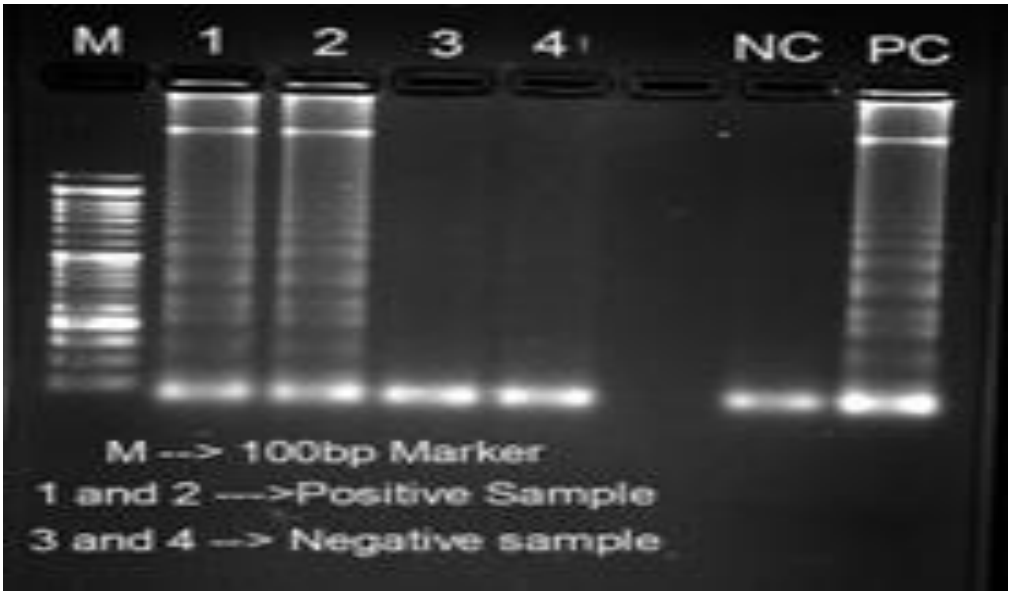


Figure – 15: Positivity of LAMP results in all genes (56kDa/47kDa and groEL) vs IgM ELISA and IgM IFA (n=280)

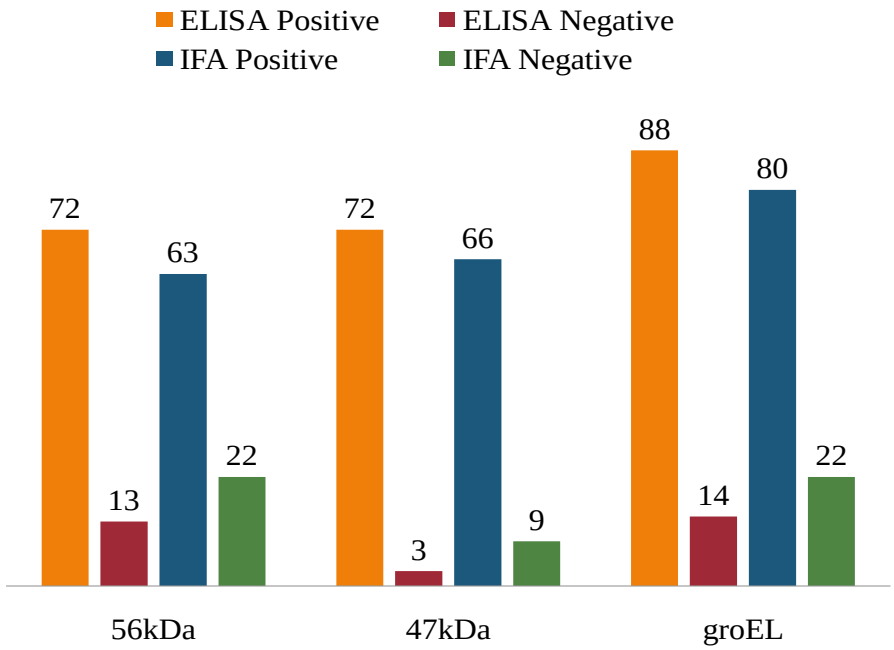


Table – 5: Positivity of 56kDa/47kDa/groEL for LAMP in ELISA and IFA (n =280)

Name of the gene targets	LAMP				LAMP			
	ELISA Positive (n = 140)	**ELISA Negative (n = 140)**	**Total**	**p value**	**IFA Positive (n = 136)**	**IFA Negative (n = 144)**	**Total**	**p value**
56kDa	85	17	102	**0.0131**	78	24	102	0.0660
47kDa	73	10	83	**0.0003**	64	19	83	**0.0167**
groEL	83	12	95	**0.0014**	63	32	95	0.2459

* p value <0.05 was considered as statistically significant

Results of molecular diagnostic tests

Positivity of three different genes in one or more molecular tests is projected in as histogram (Figure – 16). Positivity among molecular tests N-PCR, qPCR and LAMP for one or more gene targets (56kDa, 47kDa and LAMP) is shown in Figure – 17.

Figure – 16: Positivity of three different genes in one or more molecular tests (n=280)

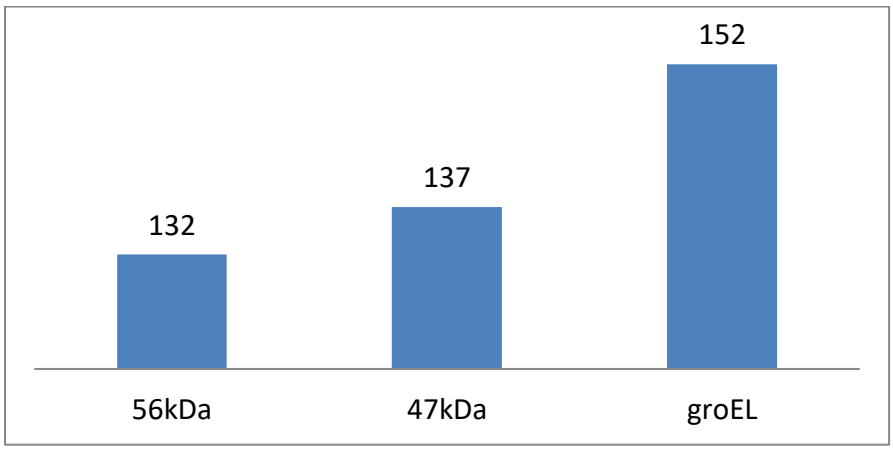
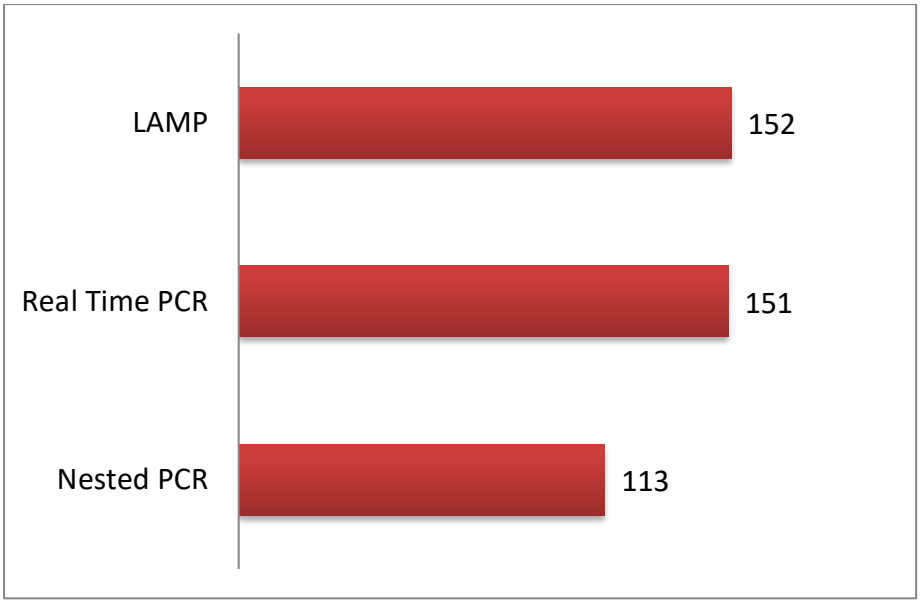


Figure – 17: Positivity among molecular tests N-PCR, qPCR and LAMP for one or more gene targets (56kDa, 47kDa and LAMP) (n=280)



Results of different combination of three molecular tests for 56kDa, 47kDa and groEL genes (n=280)

Overall positivity was observed in 132 (47.14%), 137 (47.93%), 152 (54.29%) for 56kDa, 47kDa and groEL gene respectively. Different combination of three molecular tests with the gene targets were detailed in Table – 6.

Table – 6: Comparison of Nested PCR, Real–Time PCR and LAMP results for 56kDa, 47kDa and groEL genes (n=280)

Category	Positive for 56kDa only	Positive for 47kDa only	Positive for groEL only
Nested PCR only	7	17	12
qPCR only	18	19	24
LAMP only	32	26	20
Nested PCR + qPCR	5	18	21
qPCR + LAMP	37	16	19
Nested PCR + LAMP	8	19	18
Nested PCR + qPCR + LAMP	25	22	38
Total Positive	132 (47.14%)	137 (47.93%)	152 (54.29%)

Table – 7 compares the results of individual molecular tests for 56kDa/47kDa/groEL genes

Table – 7: Comparison of molecular tests results for all three genes

Category	Number positive in N-PCR (%)	Number positive in qPCR (%)	Number positive in LAMP (%)
Positive for all three gene targets: 56kDa + 47kDa + groEL	23 (8.21%)	26 (9.28%)	34 (12.14%)
Negative for all three gene targets: 56kDa + 47kDa + groEL	167 (59.64%)	129 (46.07%)	128 (45.71%)
Concordance (all three genes positive + all three genes negative)	190 (67.85%)	155 (55.35%)	162 (57.85%)

Table – 8 compares three individual gene target results in molecular tests N-PCR + qPCR + LAMP

Table – 8: Comparison of three gene targets results in molecular tests

Category	Number positive for 56kDa (%)	Number positive for 47kDa (%)	Number positive for groEL (%)
Positive in all three tests: N-PCR + qPCR + LAMP	25 (8.93%)	22 (7.86%)	38 (13.57%)
Negative in all three tests: N-PCR + qPCR + LAMP	68 (24.29%)	73 (26.07%)	58 (20.71%)
Concordance (all molecular tests positive + all molecular tests negative)	93 (33.21%)	95 (33.93%)	96 (34.28%)

Clinical and Laboratory Findings:

An analysis of clinical features and laboratory findings of 184 ST positive cases (children and adults) out of 210 patients is presented in Table – 9.

Table – 9: Clinical findings and Laboratory results of Scrub Typhus cases confirmed by Serology and/or Molecular tests : Comparison between Children and Adults (n = 184)

Clinical/Laboratory findings	Children (0-18years) (n=64)	Adults (≥ 19 years) (n=120)	Total (n=184)(%)	p values*
Fever ≤ 7 days	28	76	104 (56.52)	**.010705**
Fever ≥ 7 days	36	44	80 (43.48)	**.010705**
Chills & Rigor	34	82	116 (63.04)	**.041793**
Myalgia	22	41	63 (34.24)	.977371
Headache	25	91	116 (63.04)	**.000001**
Cough & Expectoration	42	94	136 (73.91)	.061516.
Abdominal Pain	29	72	101 (54.89)	.056528
Hepatomegaly	13	25	38 (20.65)	.933754
Splenomegaly	5	12	17 (9.24)	0.7911
Malaise	19	44	63 (34.24)	.341994
Nausea	23	52	75 (40.76)	.330865
Vomiting	20	53	73 (39.67)	.088053
Pneumonitis	5	13	18 (9.78)	0.6090
Eschar	13	19	32 (17.39)	.445185
Rash	2	3	5 (2.71)	1.0000
Lymphadenopathy	9	21	30 (16.30)	0.6953
Pedal edema	0	5	5 (2.71)	0.1649
↓ Platelet Count (≤1.5 Lakhs)	13	26	39 (21.20)	.830496
↑Increased Liver Enzymes	9	16	25 (13.59)	0.8906

(AST/ALT/ALP)[£]				
Creatinine (>1.0)	7	20	27 (14.67)	0.4081
↑ Leucocytosis (>11,000 Cu mm)	8	23	3 (1.63)	0.3452

*p values <0.05 were considered significant; [£] AST – Aspartate Transaminase/ALT – Alanine Transaminase/ALP – Alkaline Phosphatase.

6.8. Gene Sequencing:

The specificity of the sequences was determined by BLAST (Basic Local Alignment Search Tool) analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and it shows 99-100% similarity with the other isolates available in the National Center for Biotechnology information (NCBI) database. These bidirectional sequences were aligned using biological sequence alignment editor called (MultAlin) software. Ninety six nucleotides were aligned with corresponding reference sequences, obtained from Gen Bank and phylogenetic tree was constructed for three genes (Figures - 18,19 & 20) using by Maximum Likelihood method by using MEGA software Version 10.0.

The aligned sequences were submitted to Gen Bank and the sequences were published with accession numbers MG601875 to MG601917 for 56kDa, MG601918 to MG601959 for groEL gene and MG601960 to MG601969 for 47kDa of *O. tsutsugamushi*, respectively. Additionally, one more sequence was submitted to GenBank and was provided accession number MH758790 for groEL gene (yet to be published).

6.9. Phylogenetic Tree:

Phylogenetic tree of 56kDa forms four lineages (Figure – 18). The first lineage which is supported by a bootstrap value of 99 % consists of eight sequences from this study. The isolates JA9716, M1417, OT4713 and M1616 are arranged in a clade along with the reference strain ISS – 2 reported from Madhya Pradesh, India. The study sequences JA 14316

(2) and JA2016 are more closely related to each other, supported by a bootstrap value of 94 %. Similarly, isolates JA9916 and FB2615 are more closely related with boot strap value of 99 %. Thirteen study sequences (N5915, D3415, N10815, OT8715, AP2915, JA9515, D8415, D13015, JA3116, JA5316, M1116, JA14116, N8915) in the second lineage were closely arranged with the strains earlier reported from the regions of Pondicherry (Union Territory) and Manipal (India). The study sequence ST1315 forms another separate lineage (third) along with reference sequences (MG283163, MG283194) obtained from Manipal which is highly supported by a bootstrap value of 99 %. The fourth larger lineage arranged in two clades is highly supported by a bootstrap value of 94 % and 90 %. The sub clade which is supported by a bootstrap value of 99 % has five study sequences (JA4915, N6715, D5915, D10615, FB4816). First clade has five of our sequences and is arranged more closely with the reference strains reported from Vellore, Tamil Nadu, India and Thailand. Another larger sub clade is divided further into two sub clades in which three study sequences (N3215, D3315 and D5315) are closely arranged with a bootstrap value of 61 %. The next sub clade has 13 study sequences (N9015, D7015, JA10715, D8915, D9515, D6115, D14115, JA4216, D10515, OT413, JA1316, JA14716, M1516) which are grouped closely with the Karp reference sequences reported from UK. The constructed tree is highly supported by a bootstrap value greater than >90% making the tree highly reliable.

In groEL tree (Figure - 19), the study sequences shares the common ancestor with the reference strain (KC68834). Branching pattern of strains observed in the first lineage is highly supported by a boot strap value of 96%. The 17 of our study sequences (S4013, JA2616, JA15316, N0915, JA15416, JA12716, JA12416, JA12316, JA3316, JA6416, JA5916, D8315, D7215, JA1616, D6915, ST3015 and D2615) in the pattern which is arranged in sub clades 1 and 2 are grouped along with the reference sequences which were reported from Korea and China. The other five study sequences (JA7615, JA5516, JA8816,

N8515 and JA9914) in the second lineage are more closely arranged with the reference strain reported from China and it has a boot strap value of 98%. The third lineage has three of our study sequences (JA12916, JA5716 and JA0916) are very closely related to each other with a boot strap value of 63%. The fourth lineage consists sixteen of our study sequences. Herein three of our sequences (KOT0115, D12915 and D9415) are arranged in a clade within the cluster reported from Japan and Korea. Two of the study sequences S2813, FB3616 are highly aligned with each other. The other seven sequences (MH785790, JA4716, OT4713, JA7615, JA2416, JA7815 and N8815) were grouped with the strains reported from Thailand and Japan.

The phylogenetic tree of 47kDa (Figure – 20) is arranged in the form of a clade supported by a boot strap value of 38% and 79%. The clade which is supported by a boot strap value of 79% has the study sequences FB1517, FB3716 and N1113. Within which the sequences FB1517, FB3716 are closely related with a value of 80%. The clade which is supported by a boot strap of 38% has four of our sequences JA8316, FB6916, D11415 and N0513. The sub clade formed by three of our sequences (N6315, D12115, JA1816) matched with the strains reported from Uttarkhand, India.

Figure – 18: Phylogenetic analysis of *Orientia tsutsugamushi* on 56kDa gene by Maximum Likelihood method:

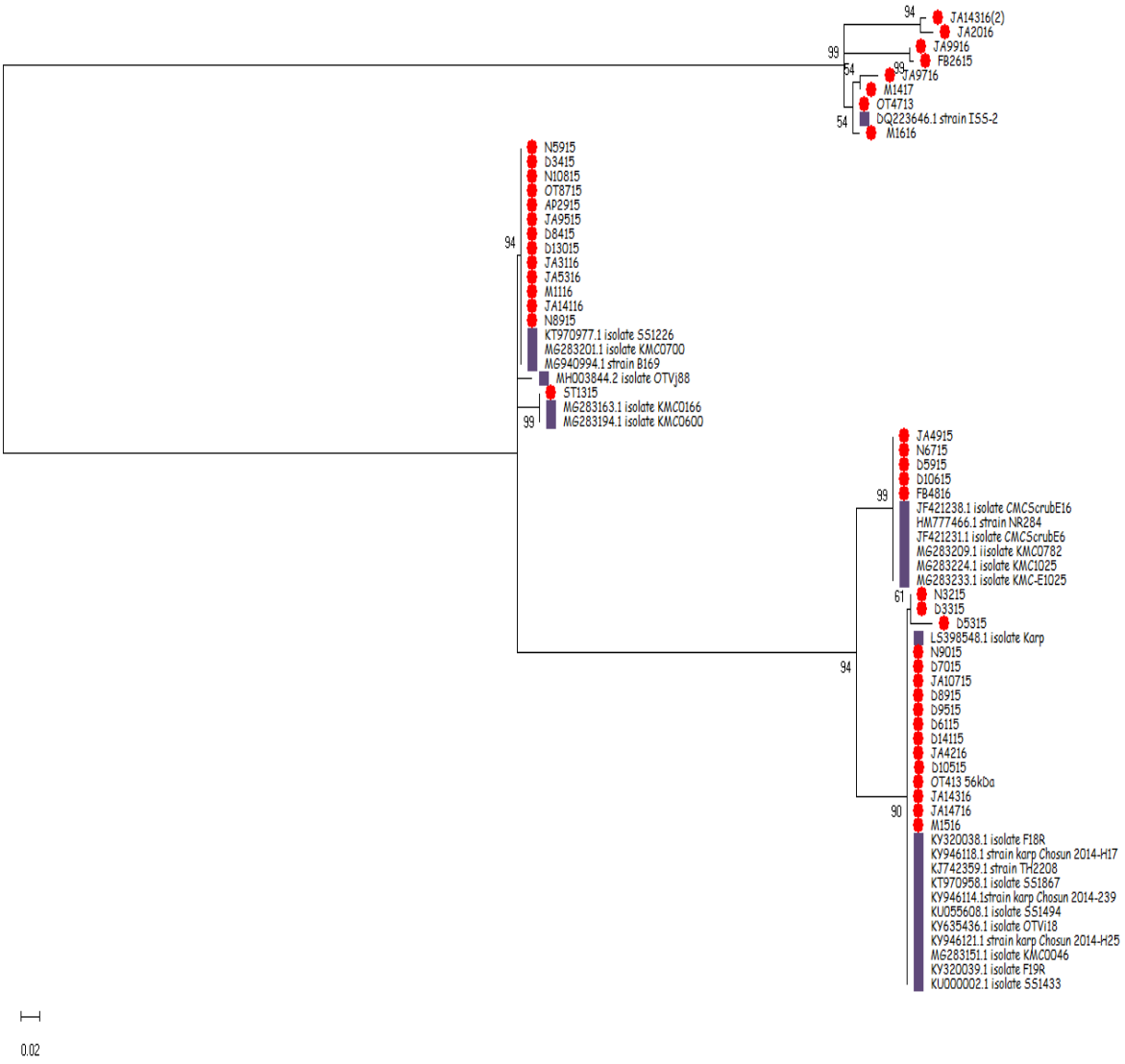


Figure – 19: Phylogenetic analysis of *Orientia tsutsugamushi* on groEL gene by Maximum Likelihood method:

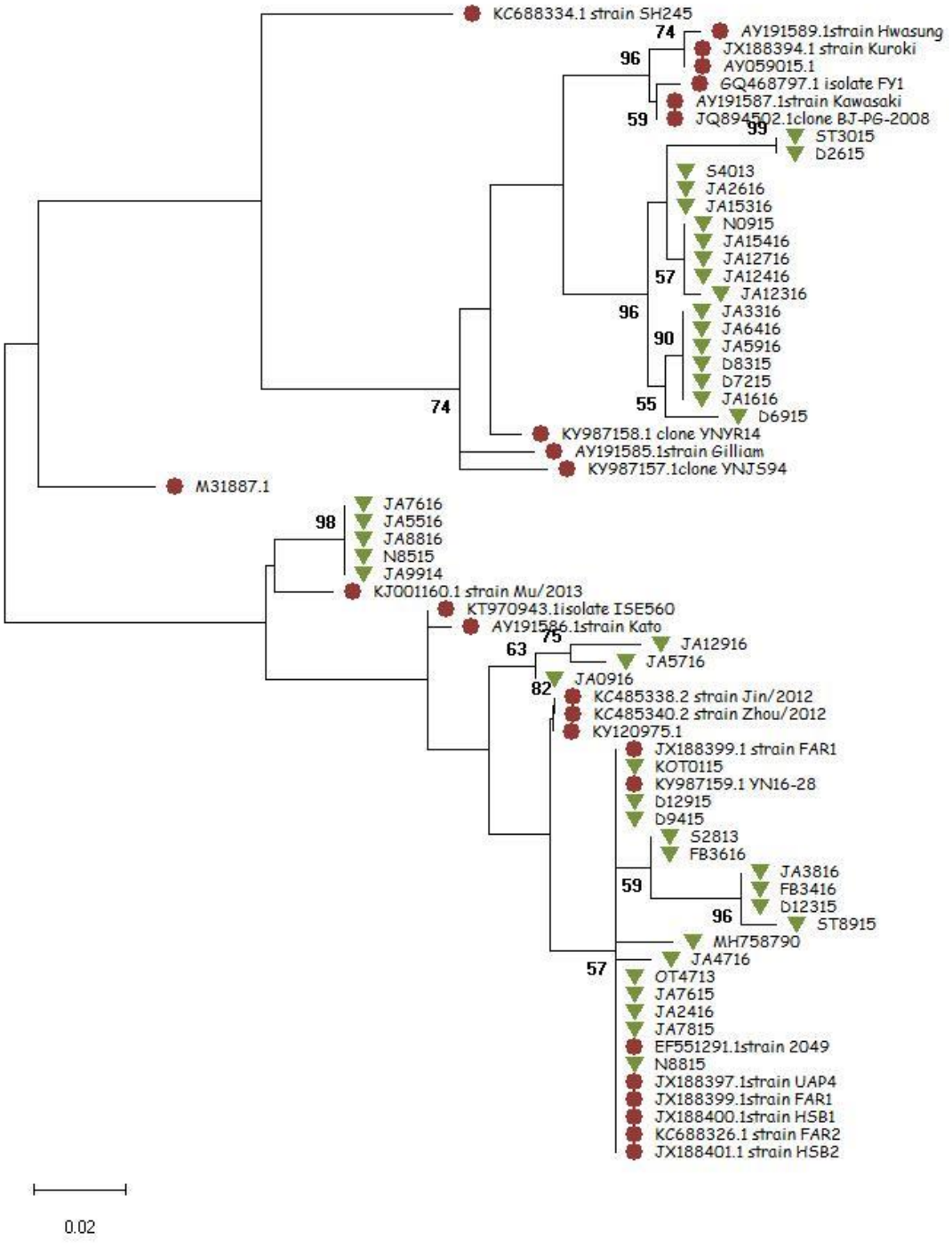
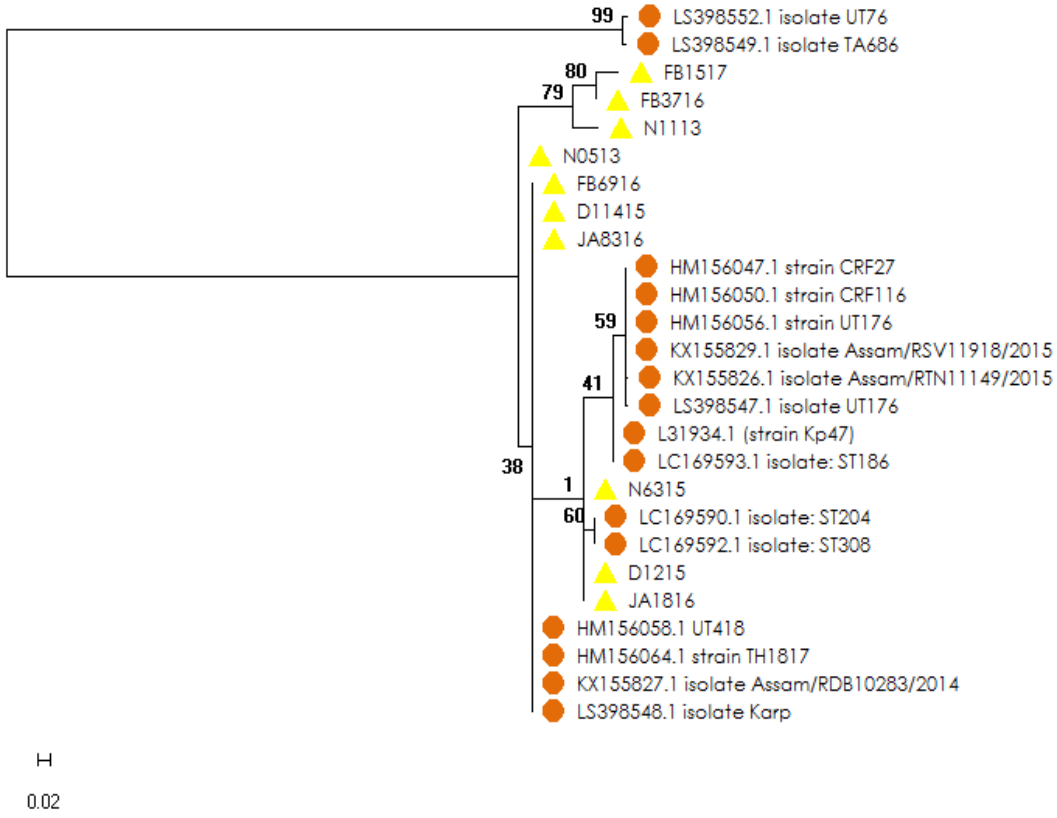


Figure – 20: Phylogenetic analysis of *Orientia tsutsugamushi* on 47kDa gene by Maximum Likelihood method:



7. DISCUSSION

7.1. ELISA vs IFA:

Sixteen IgM ELISA Negatives were positive in IgM IFA and 38 IgG ELISA negatives were positive in IgG IFA. This could be due to the difference in the antigen content of these kits. Conversely 20 IgM and 35 IgG ELISA positives were found negative in IFA. The strength of agreement between IgM ELISA and IgM IFA is considered to be 'good' with a Kappa value 0.743 with 95% confidence interval. The strength of agreement between IgG ELISA and IgG IFA is considered to be 'fair' with 95% confidence interval and Kappa value 0.280.

7.2. Paired samples in ELISA and IFA (n=58)

Acute samples of all 58 patients were positive in IgM ELISA, whereas only 49 of them were positive in IgM IFA. Regarding convalescent samples, IgM ELISA was positive in 50 patients out of whom 42 were IgM IFA positive. With reference to IgG in acute sera, a total of 25 were positive in ELISA while 33 were positive in IFA. In convalescent cases, equal numbers of 38 patients were positive in ELISA/IFA.

Considering both ELISA and IFA, IgM positivity was observed more in acute samples than convalescent samples. However for IgG, more positivity was noted in convalescent samples than acute samples. This is due to persistence of IgG longer than IgM. Concordant results for IgM positivity in acute and convalescent samples was obtained in 38 (65.52%) patients. However the concordance of patients positive for IgG was 22 (37.93% in both IFA and ELISA.

7.3. Commercial gel PCR vs N-PCR, qPCR and LAMP (56kDa) (n=280)

The overall positivity of commercial gel PCR was 8.93%, whereas N- PCR was almost double (16.07%). It might be because of different targets used, a band at 483bp was seen in Nested PCR and 473bp was observed in commercial PCR. Out of 280 samples tested, Commercial gel PCR identified 25 samples for 56kDa gene target. Among these 25, the positivity observed in other molecular methods for the same 56kDa gene was N- PCR → 8 (32%), qPCR → 14 (56%) and LAMP → 19 (76%). Purified products of these DNA extracts were sequenced and found that these two sequences are different from each other.

7.4. Comparison of molecular methods:

In the present study, while evaluating results of Nested PCR targeting three genes, the groEL gene detects more number of cases (n = 89), followed by 47kDa (n = 76) and 56kDa (n = 45). This could be due to of primers selection. Real Time PCR also picked up more positive cases in groEL gene 102/280 (36.42%), moderate in 56kDa 85/280 (30.36%) and lowest in 47kDa 75/280 (26.79%). Patricia et al., 2017 has recently reported prevalence of ST in Puducherry. Among 145 samples tested by them, 135 were positive in IgM ELISA. GroEL gene in N-PCR was positive in 68.2% and 56kDa in 27.6% patients. Our study is the first report targeting 47kDa gene of *O. tsutsugamushi* in India. As presented in Table – 7, positivity for all three genes in N-PCR, qPCR and LAMP was 8.21%, 9.28% and 12.14%. The overall concordance between these three tests was 67.85%, 55.35% and 57.85% respectively. Regarding the individual gene targets, as outlined in Table – 8, all three molecular tests were positive in 8.93%, 7.86% and 13.57% for 56kDa, 47kDa and groEL. Concordance (all positive and all negative in the three molecular tests) of 33.21%, 33.93% and 34.28% was observed for 56kDa, 47kDa and groEL.

Saisongkorh et al., 2004 suggested that Nested PCR is more sensitive than serological tests. While Nested PCR has high specificity, sensitivity is less when compared to IgM ELISA (Srinivasan et al., 2017).

Use of multiplex Real Time PCR in *O. tsutsugamushi* detection, will minimize the false positivity of ST cases, because of a single target used (Tantibhedyankul et al., 2017). They found the sensitivity and specificity of Real Time PCR assay was 86.5% and 100% respectively. By Real Time PCR 73% of positivity was detected by Bakshi et al., 2007. We have obtained a positivity of 38.89% (Anitharaj et al., 2017). In the present study, qPCR positivity for groEL gene only was 18 in ELISA positive and 11 in ELISA negative. Similarly, 56kDa alone identified 12 cases in ELISA positive and 11 cases in ELISA negative, whereas 47kDa alone identified 12 cases in ELISA positive with two in ELISA negative. Therefore we have reached the conclusion that including all three targets will increase the sensitivity of qPCR.

Compared to N-PCR and qPCR, LAMP positivity was highest in 56kDa with 36.43% (102/280), 33.93% for groEL gene (95/280) and 29.64% (83/280) for 47kDa gene. Thitimonrat Duangchanda, 2012, while targeting groEL gene had a high positivity in LAMP than the PCR.

Kelly et al., 2009 observed that the most conserved region of 47kDa (HtrA protein) detects < 21 copies of DNA in blood samples. In the present study, 56kDa in qPCR was positive for 40 samples, which were all negative in N-PCR. This might be due to the use of different primers. The positivity observed in the present study for Taq Man qPCR of 26.79% is almost similar when compared to N-PCR (27.14%) with reference to 47kDa gene. Results of N-PCR and qPCR showed higher positivity in groEL gene compared to LAMP. Conversely, LAMP positivity was more in 56kDa and this might be due to designing of primers from highly conserved region. Paris et al., 2011 demonstrated that LAMP is a comparable diagnostic assay to Real Time PCR and Nested PCR. The major drawback of LAMP is the need for optimizing $MgSO_4$ concentration of the reaction mixture on a day-to-day basis so as to obtain the desired colour change in the positive and negative samples.

Various combinations of three molecular tests for 56kDa, 47kDa and groEL genes were compared in Table – 6. Three combinations with different genes showed highest positivity viz., N-PCR + qPCR - 21 (7.5%) in groEL gene, qPCR + LAMP - 37 (13.2%) in 56kDa and N-PCR + LAMP-19 (6.79%) for 47kDa. Overall positivity of 54.29% was observed in groEL by either Nested PCR/Real Time PCR/LAMP followed by 49.29% in 47kDa and 47.14% in 56kDa respectively. Thus the sensitivities of these genes follow the following order: groEL (152) > 47kDa (137) > 56kDa (132). On the whole, the positivity was observed in molecular methods for one or more gene targets were LAMP (152) > Real Time PCR (151) > Nested PCR (137).

While comparing all three molecular methods viz., N-PCR, qPCR and LAMP for 56kDa ($p = <0.00001$) statistical significance was found. However, no statistical significance was observed among the positivity in molecular tests for the other two genes 47kDa ($p = 0.713446$) and groEL ($p = 0.510224$).

Table – 9 analyses the clinical findings and laboratory results of Scrub Typhus cases confirmed by Serology and/or Molecular tests. Comparison between Children and Adults (n = 184) revealed that more number of adults had Fever ≤ 7 days ($p = .010705$), Fever ≥ 7 days ($p = .010705$), Chills and rigor ($p = .041793$) and Headache ($p = .000001$).

7.5. Serological false positivity:

Prakash et al., 2006; Prakash et al., 2011 reported false positivity of ST IgM ELISA other diseases like malaria, pulmonary tuberculosis, typhoid fever and septicemia with viridians Streptococci. In our study, ST IgM ELISA false positivity was observed in four of those PCR negative samples, who had an infection of Typhoid and Dengue.

7.6. Phylogenetic analysis:

All sequenced samples showed 98-100% identity in NCBI blast. During the last two decades, the molecular evidence of *Orientia tsutsugamushi* DNA was proceeded by various

methods like Conventional PCR, Nested PCR, Real Time PCR and LAMP in limited countries and the sensitivity among these molecular assays varies from 8.8% - 86.5% in South Korea (Kim et al., 2006), Thailand (Tantibhedyankul et al., 2017) and India (Srinivasan et al., 2017). Serological cross reactivity of *Orientia tsutsugamushi* is mainly based on serotypes (Sando et al., 2018). Due to genetic diversification of *Orientia tsutsugamushi*, a number of recombinant genotypes were reported, such as Karp, Kawasaki, Gilliam, Kato, Boryong,TA763 (Kim et al., 2017). Karp is one of the major dominant genotype of *Orientia tsutsugamushi* varying from 40 – 64.4% and it is reported in many countries such as Vietnam, Thailand (Viet et al., 2017; Kelly et al., 2009),followed by TA763 17%, Gilliam 17% and Kato 12% (Nguyen et al., 2017). Regarding the genotypes of *O. tsutsugamushi* circulating in India, Patricia et al., 2017 from Puducherry reported higher positivity of 42.9% for Karp and 17.1% for Kato. Usha et al., 2016 from Andhra Pradesh also had a higher prevalence of Karp strains (70.4%) followed by Kawasaki (29.6%). Varghese et al., 2015 from Tamil Nadu and other parts of India observed that they have Kato as the majority strain with 61.5% followed by Karp (27.1%), Neimang (8.7%) and Ikeda (2.3%). According to Biswal et al., 2016 from Chandigarh, Boryong occupied the highest prevalence with 63.4% followed by Karp (23.6%), Gilliam (11.8%) and Kawasaki (1.2%). The present study has recorded a moderate positivity of 32.29% for Karp like strains. About 17.7% of our isolates are grouped with a Kawasaki as well as Kuroki strains. Most of our isolates has highest homology with that of the strains reported from Pondicherry and Manipal in Tamil Nadu as well as Korea, Japan and China from overseas.

7.7. Comparative cost of each serological/molecular test:

For IgM/IgG IFA, the cost is about Rs. 1500 /- per each test. Molecular tests per sample cost about Rs. 700 for N-PCR, Rs. 1000/- for qPCR and Rs. 500/- for LAMP for each gene target.

8. CONCLUSION

Comparing results of ELISA and IFA, ELISA yields higher positivity than IFA. Hence ELISA may be considered as a better alternative to the technically demanding IFA.

Results of Nested PCR showed higher sensitivity than commercial PCR (Geno-sen's). Real Time PCR by the commercial kit Geno-sen's detects more cases than N-PCR.

Even though primer designing in LAMP is quite difficult, it is uncomplicated, quick, and also it will identify more cases with fewer copies of DNA. The setback of LAMP is the frequent need for optimizing MgSO4 concentration of the reaction mixture.

O. tsutsugamushi identification by molecular methods including all three genes will result in identifying more number of positive cases. In the present study, the statistical significance was observed in 56kDa, but not in other two genes (47kDa and groEL) among the three molecular methods. To our knowledge, this is the first report in India of molecular diagnosis by targeting all three genes (56kDa, 47kDa and groEL) by N-PCR, qPCR and LAMP.

Gene sequencing result indicates that most of the sequences were related to those submitted from India as well as overseas. Few dissimilar sequences from the existing ones may belong to the newer strain and this will be helpful in future for development of vaccine.

9. LIMITATIONS OF THIS RESEARCH AND SCOPE FOR FUTURE WORK

Archived paired serum samples were available for only 58 among 210 patients. This is because of the long distance most of our patients must travel to reach us so as to provide convalescent blood samples.

Extension of the research work to cover a wider geographical area in regions surrounding Puducherry might help in identifying additional genotypes of *O. tsutsugamushi*.

Development of a Multiplex PCR targeting all three genes would help in the rapid confirmation of ST.

10. SUMMARY

1. This study has included 280 participants. Out of 140 ST IgM ELISA positive cases, 52 (37.14%) were IgG ELISA positive. Among 140 ST IgM ELISA negative serum samples, 13 (9.28%) were positive in IgG ELISA.

2. Among 140 IgM ELISA positive and 140 negative samples 120 (85.71%) and 16 (11.43%) were positive in IgM IFA respectively.

3. Regarding IgG IFA, out of 65 IgG ELISA positive 30 were positive and among 215 IgG ELISA negative cases, 38 were positive in IgG IFA.

4. Fifty eight patients provided paired blood samples. Among 58 paired sera, all acute samples were positive for IgM ELISA, but only 35 were positive in IgG ELISA. Regarding IFA, 49 were positive in IgM IFA but only 33 were positive in IgG IFA. Out of 58 convalescent samples, positivity observed was 50 in IgM ELISA and 38 in IgG ELISA. Regarding IFA, IgM IFA was positive in 42 patients and IgG IFA was present in 38 patients. Concordant results for IgM positivity between IFA and ELISA in both acute and convalescent samples was 38 (65.52%). However the concordance between IFA and ELISA in both acute and convalescent samples for IgG was 22 (37.93%).

5. Regarding molecular diagnostic tests, in-house Nested PCR positivity of 16.07% was observed for 56kDa, 27.14% for 47kDa and 31.79% for groEL.

6. In commercial gel PCR (Geno-sen's) targeting 56kDa gene, only 25 were (8.93%) positive. Among these 25, the positivity observed in other molecular methods for the same 56kDa gene was N- PCR → 8 (32%), qPCR → 14 (56%) and LAMP → 19 (76%). Purified products of these DNA extracts were sequenced and found that these two sequences are different from each other.

7. Real Time PCR (qPCR) positivity of genes targeting 56kDa, 47kDa and groEL were 30.36%, 26.79% and 36.43% respectively.

8. The primers for LAMP targeting 56kDa of *O. tsutsugamushi* were successfully designed using Hualien – 5 strain (Accession no. AY243357.1) and utilized for the research. The positivity of LAMP in 56kDa was 36.43%, followed by 29.64% in 47kDa and 33.93% in groEL gene.

9. To our knowledge, this is the first report of LAMP targeting *O. tsutsugamushi* by three genes such as 56kDa, 47kDa and LAMP respectively.

10. Nested PCR and Real Time PCR has higher positivity in groEL gene, Whereas, LAMP detected more cases in 56kDa.

11. The overall positivity of gene targets observed in any one of the molecular methods (N-PCR/qPCR/LAMP) was groEL – 152, 47kDa – 137 and 56kDa – 132.

12. The overall positivity of molecular methods observed in any one of the gene targets (56kDa/47kDa/groEL) was LAMP – 152, qPCR – 151 and N-PCR – 113.

13. While comparing all three molecular methods viz., N-PCR, qPCR and LAMP statistical significance was found for 56kDa ($p = <0.00001$). However, no statistical significance was observed among the positivity in molecular tests for the other two genes 47kDa ($p = 0.713446$) and groEL ($p = 0.510024$).

14. The study sequences were published in NCBI database with accession numbers MG601875 to MG601917 for 56kDa, MG601918 to MG601959, for groEL gene and MG601960 to MG601969 for 47kDa of O. *tsutsugamushi* respectively. Additionally, one more sequence was submitted to GenBank and was provided accession number MH758790 for groEL gene (yet to be published). Our study sequences have 98-100% maximum identity with the other reference strains in NCBI database. Phylogenetic tree is highly supported by a bootstrap value greater than > 90% making the tree highly reliable. Phylogenetic analyses of

this study indicates, 32.29% of our sequences are Karp- like strains, 17.7% sequences are related to Kawasaki and Kuroki strains. Remaining isolates are clustered with reference strains from India, Japan, China, South Korea and Thailand.

16. Since each of the three molecular tests, N-PCR, qPCR and LAMP need to target three different genes, a Multiplex PCR incorporating all the three genes in one would be the need of the hour, which will reduce the cost and turnaround time. However, this is not feasible for LAMP.

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12. LIST OF PUBLICATIONS FROM Ph.D WORK

1. Anitharaj V, Stephen S, Pradeep J, Park S, Kim SH, Kim YJ, Kim EY, Kim YW. Serological Diagnosis of Acute Scrub Typhus in South India: Evaluation of InBios Scrub Typhus Detect IgM Rapid Test, and Comparison with other Serological Tests. J Clin Diag Res. 2016;10(11):7-10.

2. Anitharaj V, Stephen S, Pradeep J, Pooja P, Preethi S. Diagnosis of acute scrub typhus in South India: Validation of Geno-Sen's scrub typhus real time polymerase chain reaction kit by its comparison with a serological test (Enzyme-linked immunosorbent assay). J Global Infect Dis. 2017;9:108-112.

3. Anitharaj V, Stephen S, Pradeep J, Gunasekaran D, Jayasingh K, Shanmugam J. Scrub Typhus in Pondicherry: Meta-analysis of cases reported in a Tertiary Care Teaching Hospital during 2012-2016. *Biomedicine.* 2017;37(2):221-225.

4. Stephen S, Gunasekaran D, **Anitharaj V**. Application of Enzyme Linked Immunosorbent Assay (ELISA) and Indirect Fluorescent Antibody Test (IFA) for Serodiagnosis of Acute Scrub Typhus in and Around Puducherry. *J Clin Diag Res.* 2018;12(11): DC12-DC16.

13. CONFERENCE PRESENTATIONS (NATIONAL AND INTERNATIONAL)

1. Paper presented as **Podium Presentation** at **UGC Sponsored National Conference** on 'Emerging Infectious Diseases and curative measures' at Kamarajar College, Thoothukudi, held on 22 and 23 December, 2016.

Title: 'Comparison of Serological and Molecular diagnosis for acute Scrub Typhus in Puducherry'

2. Presented **Poster in International conference** on "Microbiome in Health, Disease and Environment" held during 3rd & 4thMarch, 2017 at CIDRF,MGMCRI, Puducherry.

Topic: Molecular detection of Orientia tsutsugamushi DNA in Scrub Typhus IgM ELISA antibody positive patients by using Nested PCR assay.

3. **Oral Presentation** in **National conference** on "National Conference on Environment, Health and Disease Theme: Ecogenetics and Toxicogenomics" organized by Central Inter-Disciplinary Research Facility (CIDRF), Sri Balaji Vidyapeeth University, Puducherry on 12th February 2018.

Title: Scrub Typhus in Puducherry: Increasing the diagnostic accuracy by performing Scrub typhus IgM ELISA and Nested PCR targeting three different genes of *Orientia tsutsugamushi*